Structure
Ways & Means

Preparation of Distinct Ubiquitin Chain Reagents of High Purity and Yield

Ken C. Dong,[1,4] Elizabeth Helgason,[2,4] Christine Yu,[1,4] Lilian Phu,[3] David P. Arnott,[3] Ivan Bosanac,[1] Deanne M. Compaan,[1] Oscar W. Huang,[2] Anna V. Fedorova,[2] Donald S. Kirkpatrick,[3] Sarah G. Hymowitz,[1,*] and Erin C. Dueber[2,*]
[1]Department of Structural Biology
[2]Department of Early Discovery Biochemistry
[3]Department of Protein Chemistry
Genentech, 1 DNA Way, South San Francisco, CA 94080, USA
[4]These authors contributed equally to this work
*Correspondence: hymowitz@gene.com (S.G.H.), dueber.erin@gene.com (E.C.D.)
DOI 10.1016/j.str.2011.06.010

SUMMARY

The complexity of protein ubiquitination signals derives largely from the variety of polyubiquitin linkage types that can modify a target protein, each imparting distinct functional consequences. Free ubiquitin chains of uniform linkages and length are important tools in understanding how ubiquitin-binding proteins specifically recognize these different polyubiquitin modifications. While some free ubiquitin chain species are commercially available, mutational analyses and labeling schemes are limited to select, marketed stocks. Furthermore, the multimilligram quantities of material required for detailed biophysical and/or structural studies often makes these reagents cost prohibitive. To address these limitations, we have optimized known methods for the synthesis and purification of linear, K11-, K48-, and K63-linked ubiquitin dimers, trimers, and tetramers on a preparative scale. The high purity and relatively high yield of these proteins readily enables material-intensive experiments and provides flexibility for engineering specialized ubiquitin chain reagents, such as fluorescently labeled chains of discrete lengths.

INTRODUCTION

Protein ubiquitination serves as a critical regulatory mechanism for a wide array of cellular processes (Pickart and Fushman, 2004). Posttranslational modification of a target protein by the small, 76 amino acid ubiquitin molecule occurs through a multistep enzymatic cascade that includes an ATP-dependent E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme and an E3 ubiquitin ligase, culminating in the attachment of the ubiquitin C terminus to the ε-amino group of a primary amine, generally a lysine residue. While some E3 ligases, such as the HECT family, directly couple ubiquitin to the substrate, others act to pair protein targets with E2 enzymes that catalyze the ubiquitin attachment. In either case, an E3 ligase drives substrate selection, with ubiquitination of a single lysine (monoubiquitination) versus multiple lysine residues (multimonoubiquitination) potentially leading to differential cellular fates for a single protein target (Harper and Schulman, 2006; Kirkpatrick et al., 2006).

Further signal complexity arises from the extension of ubiquitin polymers on substrate proteins, which can assemble via any one of the seven ubiquitin lysine residues, or N terminus. Although the functional consequences of each polyubiquitin chain type are still being elucidated, clear cellular outcomes have been observed for the most abundant chain linkages. For example, ubiquitin chains conjugated through lysine 48 (K48-linked) are well characterized to promote proteasomal degradation of the modified protein (Chau et al., 1989; Ikeda and Dikic, 2008). K11-linked ubiquitin tags have also been shown to act as degradation signals for substrates of the anaphase promoting complex (APC) in cell division regulation, whereas K63-linked polyubiquitin chains have been best studied in their nonproteolytic roles in proinflammatory signaling and DNA repair pathways (Baboshina and Haas, 1996; Chen and Sun, 2009; Jin et al., 2008; Kirkpatrick et al., 2006). The biology of other linkages, such as linear ubiquitin, is becoming better defined with continued research (Gerlach et al., 2011; Ikeda et al., 2011; Ikeda and Dikic, 2008; Iwai and Tokunaga, 2009; Jin et al., 2008; Tokunaga et al., 2011; Virdee et al., 2010; Xu et al., 2009).

Indeed, current research in the field aims to elucidate the molecular mechanisms by which these diverse polyubiquitin signals are assembled and interpreted by cellular receptors. These efforts were greatly aided by the discovery that select E2 enzymes are capable of catalyzing the formation of polyubiquitin chains of specific linkages in vitro, without the presence of an E3 ligase (Chen and Pickart, 1990; Chen et al., 1991; Wu et al., 2010). In addition to identifying simplified systems with which to probe the mechanism of ubiquitin chain formation, these findings also provided a means of preparing free (i.e., uncoupled to substrate) polyubiquitin chains that can be used to evaluate how ubiquitin-binding proteins distinguish between distinct polyubiquitin signals.

Initial chain synthesis protocols for K48- or K63-linked chains of defined length were particularly focused on the preparation of ubiquitin dimers (or longer constructs) that had distinguishable distal and proximal protomers for NMR studies and relied on successive rounds of conjugation and deblocking steps, using mixed populations of lysine-blocked and C-terminally blocked mutants, to force protomer orientation (Piotrowski et al., 1997;

Pickart and Raasi, 2005; Varadan et al., 2002a). While these methods remain useful for differentially labeling ubiquitin protomers within a chain or for making defined linkage chains (Blankenship et al., 2009; Newton et al., 2008; Reyes-Turcu et al., 2008; Varadan et al., 2004, 2005), one can also use wild-type ubiquitin stocks and specific E2 enzymes to produce milligram quantities of ubiquitin homopolymers of discrete lengths in a one-step synthesis reaction (Cook et al., 1992, 1994). Such reagents have been used to study facets of polyubiquitination such as the inherent structure of different ubiquitin polymer types, ubiquitin chain recognition by a variety of ubiquitin-binding proteins, and the activity and specificity of deubiquitinating (DUB) proteases that disassemble and regulate polyubiquitin signals (Bosanac et al., 2010; Dynek et al., 2010; Eddins et al., 2007; Komander et al., 2009; Lo et al., 2009; Rahighi et al., 2009; Varadan et al., 2002b). Efforts to further understand the detailed molecular mechanisms of these and other aspects of polyubiquitin signaling would benefit greatly from the availability of methods to readily generate wild-type and mutant polyubiquitin reagents that are of high purity and in greater quantities than are typically used on a biochemical scale.

Here, we describe optimized methodologies for preparing high-quality linear, K11-, K48-, and K63-linked polyubiquitin chains in sufficient quantities to enable structural and biophysical studies, which can be readily implemented in most biophysics/structure-based laboratories (Bosanac et al., 2010; Matsumoto et al., 2010). Moreover, this custom synthesis of ubiquitin polymers allows for the incorporation of specific ubiquitin mutations that can be used to probe intra- and intermolecular interactions and which we utilize here to create site-specific, fluorescently labeled, polyubiquitin chain reagents.

RESULTS

Preparation of Recombinant Ubiquitin and Ubiquitination Enzymes

Bacterial expression of recombinant human ubiquitin (Ub; M1-G76) with an N-terminal, thrombin-cleavable His_6-tag typically yields 80–100 mg of Ub per liter of culture after purification by immobilized metal affinity chromatography (IMAC) and size exclusion chromatography. Similar amounts of linear dimeric (di-Ub_{Linear}; M1-G152), trimeric (tri-Ub_{Linear}; M1-G228), and tetrameric (tetra-Ub_{Linear}; M1-G304) ubiquitin chains are produced using this same expression and purification scheme (Figure 1A and Experimental Procedures). While Ub remains stable at 4°C, the linear ubiquitin chains are flash frozen in liquid nitrogen and stored at −80°C to avoid the gradual breakdown into individual Ub monomers that is otherwise observed (data not shown) and likely caused by trace protease contaminants.

For the synthesis of isopeptide-linked ubiquitin chains, we prepare E1 and E2 enzymes using the bacterial expression constructs detailed in Figure 1A. Specifically, we utilize human E1 activating enzyme (UBE1; M1-R1058) for all syntheses and the E2 enzyme Cdc34 (M1-S236) and the E2 heterodimer, composed of Uev1a (M1-N170) and UbcH13 (M1-I152) proteins, for generating K48- and K63-linked chains, respectively. We employ both a full-length and C-terminally truncated version of the E2 conjugating enzyme Ube2S (M1-L225; Ube2SΔC, M1-G156) for synthesis of K11-linked ubiquitin chains. All of the ubiquitination enzymes are affinity-purified according to their specific N-terminal affinity tag (see Figure 1A and Experimental Procedures) and quantified by A_{280} measurements under native conditions using calculated molar absorptivity values (ProtParam, SwissProt). Aliquots are then flash frozen in liquid nitrogen and stored at −80°C. Multiple freeze-thaw cycles are avoided for maximal activity. Working stocks of Ub that are to be used for chain synthesis reactions are also quantified by native A_{280} measurements. However, as there can be significant error in these values given the small molar extinction coefficient of Ub (ε = 1490 M^{-1} cm^{-1}), we determine the concentration of final Ub and ubiquitin chain stocks that are to be used in formal experiments by CB X Protein Assay (G-Biosciences).

Ubiquitin Chain Synthesis and Purification

Prior to preparative chain synthesis, we first assess the activity of our various ubiquitination enzyme stocks in order to better optimize ubiquitin chain production. To evaluate E1 activation of Ub, we monitor ubiquitin-loading of UBE1 in the presence of ATP and Mg^{2+}. Under these conditions, active UBE1 requires two equivalents of Ub to form a single covalent, thioester linkage to the C terminus of one of the Ub molecules, which can be detected as a band shift on a nonreducing SDS-PAGE gel. Instead of using traditional radiolabeled Ub stocks, we utilize fluorescein-labeled Ub (Invitrogen) to observe the shift in Ub mobility over a range of UBE1 concentrations (Figure 1B). We can also monitor these same E1 activation reactions using fluorescence polarization (FP), which is sensitive to the noncovalent binding of the second Ub molecule, in the form of an adenylated intermediate, in addition to the formation of the covalent Ub attachment (Figure 1C). Accordingly, these measurements reveal 1:1 and 2:1 stoichiometries for Ub:UBE1 by band shift and FP assays, respectively (Figures 1B and 1C). Together, these experiments verify that both active sites on UBE1 are competent to activate Ub and that approximately 100% of the prepared stock is functional after one freeze-thaw cycle.

Next, we evaluate the ability of the various E2 conjugating enzyme stocks to catalyze ubiquitin chain formation in small-scale test reactions. Here, we add increasing amounts of an E2 enzyme to UBE1 activation reactions where a fraction of the Ub is fluorescein labeled. After overnight incubation, the reactions can be run out on a reducing SDS-PAGE gel and the fluorescently labeled Ub and polyubiquitin species can be imaged and quantified to determine E2 enzyme concentrations that give maximum chain production (see Figure S1 available online). Alternatively, a less explicit evaluation of these reactions by FP measurements (Figure 1D) provides comparable results with minimal time investment. Except for the inclusion of fluorescein-labeled Ub, these test conditions mirror those used for wild-type preparative chain synthesis (i.e., all reaction components are at preparative-scale concentrations); however, the reaction volumes are kept much smaller for these analytical samples. In this way, we aim to have the results of the test reactions best translate to large-scale syntheses.

Once the UBE1 stock is validated and optimal E2 concentrations are determined, we proceed to assembling the preparative Ub chain reactions. We typically react 50 mg of Ub with UBE1 and the appropriate E2 enzyme overnight in E1 activation buffer.

A

Summary table of protein reagents

protein	boundaries	expression vector	affinity tag	MW (-tag)	description
Ub	M1-G76[a]	pET-15b	His_6-thrombin site	8.8 kDa	"WT" Ub monomer
di-Ub_{Linear}	M1-G152[a]	pET-15b	His_6-thrombin site	17.6 kDa	Ub dimer (linear)
tri-Ub_{Linear}	M1-G228[a]	pET-15b	His_6-thrombin site	26.4 kDa	Ub trimer (linear)
tetra-Ub_{Linear}	M1-G304[a]	pET-15b	His_6-thrombin site	35.2 kDa	Ub tetramer (linear)
Ub-M1C	C1-G76[a]	pET-15b	His_6-thrombin site	8.8 kDa	labeling-competent Ub mutant
UBE1	M1-R1058	pGEX6P-1	GST-PreScission site	118 kDa	E1 activating enzyme
Cdc34	M1-S236	pGEX6P-1	GST-PreScission site	27 kDa	E2 conjugating enzyme(K48)
Uev1a	M1-N170	EitNTH[b]	His_6-TEV site	20 kDa	E2 conjugating enzyme (K63)
UbcH13	M1-I152	EitNTH[b]	His_6-TEV site	17 kDa	E2 conjugating enzyme (K63)
Ube2S	M1-L225	pST239	Unizyme	26 kDa (+tag)	E2 conjugating enzyme (K11)
Ube2SΔC	M1-G156	pST239	Unizyme	20 kDa (+ tag)	E2 conjugating enzyme (K11)[c]

[a] All Ubiquitin species contain an N-terminal "GSH" artifact post-thrombin cleavage.
[b] EitNTH is a modified pET-52b expression vector (Novagen).
[c] Less-processive Ube2S truncation mutant.

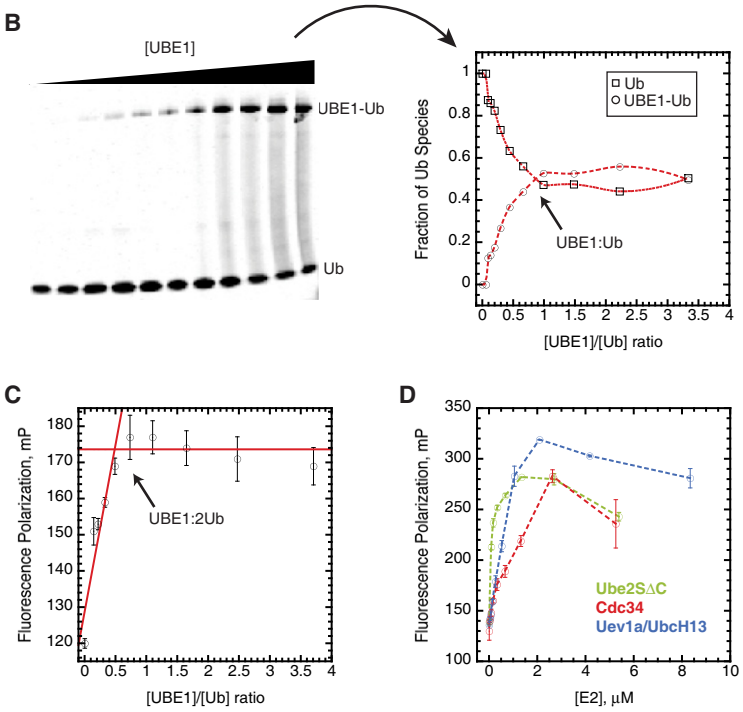

Figure 1. Validating Enzyme Stocks and Optimizing Reaction Conditions

(A) List of construct boundaries, expression vectors, affinity tags, and molecular weights for the recombinant proteins described in this work.

(B) UBE1-Ub complex formation visualized by fluorescent imaging of samples separated by a nonreducing SDS-PAGE gel (left panel). Quantification of band intensities (right panel) illustrates how increasing amounts of UBE1 band shifts fluorescein-labeled Ub to form a 1:1 covalent complex.

(C) Analysis of similar samples by FP reveals a 2:1 complex due to a second, noncovalent Ub-binding event.

(D) Addition of E2 conjugating enzymes to Ube1-Ub samples enables ubiquitin chain synthesis, leading to increased FP values as labeled Ub is incorporated into higher molecular weight polymers. Titration of various E2 stocks helps determine optimal E2 enzyme concentrations for preparative synthesis reactions.

Supporting information is found in Figure S1. In (C) and (D), FP measurements represent the mean ± the standard deviation of triplicate measurements.

Chain synthesis is confirmed by nonreducing SDS-PAGE (see Experimental Procedures) as shown in Figure 2A. Successful reactions achieve ≥50% conversion of Ub to ubiquitin chains, with ubiquitin dimer being the most abundant species after free monomer. While these preparations may seem relatively inefficient, it is important that the amount of free Ub remains above ~30% of the total ubiquitin in a reaction in order to avoid potential cyclization of ubiquitin polymers (Yao and Cohen, 2000). After verification of chain synthesis, the reactions are quenched with the addition of 20 volumes of ammonium acetate buffer to lower the pH and salt concentration of the samples. Under these acidic conditions, we can effectively separate Ub and discrete polyubiquitin chains from one another, as well as from the enzymatic components of the reaction, using cation exchange chromatography (Figure 2B). Subsequent purification of each individual chain species by size exclusion chromatography allows for further separation of the distinct polyubiquitin chain lengths (Figure 2C). As described for Ub and linear polyubiquitin stocks, all chains are then quantified by CB × protein assay and stored at −80°C.

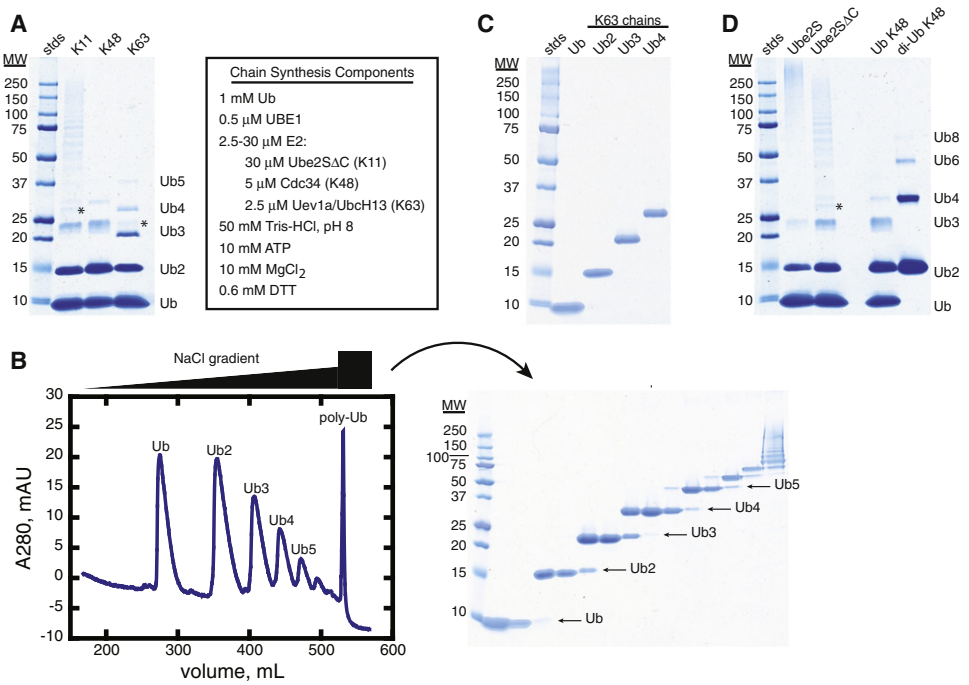

Figure 2. Preparative Ub Chain Synthesis and Purification

(A) K11-, K48-, and K63-linked chain synthesis reactions evaluated by SDS-PAGE (see Experimental Procedures). Ubiquitin polymers are simply abbreviated as Ub(n) for visual clarity (e.g., di-Ub is Ub2, tri-Ub is Ub3, etc.). Asterisks denote small amounts of E2-Ub conjugates visible in the reaction.

(B and C) (B) Mono-S chromatography of a K63-linked chain synthesis reaction separates different length chain products, as illustrated by SDS-PAGE analysis of the eluted peaks. Further purification by size exclusion chromatography results in (C) highly pure K63-linked, polyubiquitin stocks of discrete lengths. Similar results achieved with K11- and K48-linked chains are not shown.

(D) The Ube2SΔC truncation mutant shifts reaction products toward more intermediate-length K11-linked chains as compared with the full-length Ube2S enzyme. Furthermore, the yield of tetrameric and hexameric ubiquitin chains substantially improves when using dimeric starting material in chain synthesis reactions, as demonstrated with a di-Ub_{K48} stock.

Supporting information is found in Figure S2.

Depending on the amount of polyubiquitin required for a given study, additional rounds of synthesis can be carried out using the unincorporated Ub recovered from the initial preparative reaction, and/or previously unused Ub stocks, until enough polyubiquitin is prepared. When tetrameric or hexameric chains are the desired product, we find it advantageous to conduct a second round of synthesis using the appropriate ubiquitin dimer species as the input material. Doing so significantly increases the yield of these longer polyubiquitin species as compared with reactions that use Ub monomer as the basic building block (Figure 2D). Because monomeric and dimeric ubiquitin species are well resolved by the cation exchange protocols we describe, it is unnecessary to polish these stocks by gel filtration prior to use in further preparative reactions; however, special care must be taken to ensure that these acidic stocks do not alter the basic pH of the chain synthesis reactions.

In the case of K11-linked chains, initial efforts to produce significant quantities of tetramer (and trimer) species were hindered by the apparently highly processive nature of the Ube2S E2 enzyme (Garnett et al., 2009; Williamson et al., 2009; Wu et al., 2010). Although we were able to synthesize adequate amounts of K11-linked dimer, much of the remaining Ub starting material was incorporated into very high molecular weight ubiquitin polymers and Ube2S-ubiquitin conjugates as opposed to chains of intermediate size (Bosanac et al., 2010). The demonstration that a C-terminal truncation of Ube2S could decrease this observed processivity (Wu et al., 2010) lead us to create a similar deletion mutant, Ube2SΔC (residues M1–G156), for use in preparative synthesis reactions. As illustrated in Figure 2D and Figure S2, Ube2SΔC-catalyzed polyubiquitination reactions result in a more substantial accumulation of intermediately sized chains as compared with the full-length enzyme. We therefore employ the Ube2SΔC construct for producing K11 chains of discrete lengths, whereas wild-type Ube2S can be best utilized for synthesizing high molecular weight K11 chains of heterogeneous size.

Evaluation of Ubiquitin Chain Stocks

Although SDS-PAGE provides a sufficient means of monitoring the proper separation of different polyubiquitin chain lengths within a given synthesis reaction, this technique cannot effectively evaluate the homogeneity of ubiquitin linkages within a given chain population. Ubiquitin polymers can show differential mobility by SDS-PAGE depending on linkage type; however, this is a modest effect that is difficult to resolve for every chain length and linkage composition. Immunoblotting with linkage-specific antibodies provides a more accurate means of identifying particular chain types (Figure 3A). Yet, despite their proven

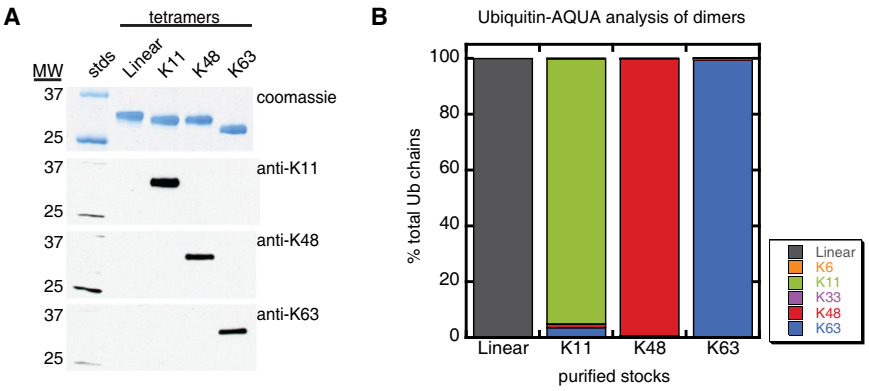

Figure 3. Evaluating the Homogeneity of Polyubiquitin Chain Stocks

(A) The linkages of purified tetramer species evaluated by western blots probed with linkage-specific antibodies. Currently, only anti-K11, anti-K48, and anti-K63 antibodies exist; thus, the linear ubiquitin tetramer in lane 2 is only visible by Coomassie staining.

(B and C) (B) Linkage profiles of purified dimers and (C) a summary table of linkage specificities for dimer and tetramer stocks as determined by ubiquitin-AQUA analysis.

Supporting information is found in Figure S3.

C

Ubiquitin-AQUA analysis of purified chain stocks

linkage type	% Target Chain	
	dimer	tetramer
Linear	100.0	99.9
K11	95.0	96.2
K48	99.2	99.2
K63	99.5	99.6

utility, linkage-specific antibodies currently only exist for K11-, K48-, and K63-linked chains (Matsumoto et al., 2010; Newton et al., 2008; Tseng et al., 2010). Thus, the remaining chain types are silent to this analysis and require comparison with immunoblots against monomeric ubiquitin to establish or exclude their presence. Furthermore, the inherent variability of the immunoblotting technique in general (e.g., differences both in overall signal strength and in the low levels of cross-reactivity with off-target proteins from one blot to another) makes this approach a qualitative, rather than a quantitative, analysis of polyubiquitin chain type.

In order to more thoroughly evaluate the homogeneity of linkages in our prepared polyubiquitin chain stocks, we employ a quantitative mass spectrometry methodology called ubiquitin-AQUA (Kirkpatrick et al., 2006; Phu et al., 2010). In this approach, we first digest polyubiquitin samples excised from SDS-PAGE gels with trypsin to cleave the chains into peptides, creating branched "–GG" signature peptides (ggSP) at sites of ubiquitin linkage. The entire pool of branched and unbranched peptides derived from the trypsin digestion is then quantified by mass spectrometry using known amounts of synthetic, isotopically labeled, internal standard peptides. Comparison to these standards allows for identification and quantification of each linkage type in a given sample. The sensitivity of this methodology enables quantification of femtomolar peptide quantities and thus provides a rigorous assessment of polyubiquitin linkage homogeneity of our prepared stocks.

Results from a typical ubiquitin-AQUA evaluation of dimer and tetramer chain stocks (including di- and tetra-Ub_{Linear} chain stocks for comparison with the synthesized isopeptide-linked chains) are summarized in Figures 3B and 3C. For di-Ub_{K11}, di-Ub_{K48}, and di-Ub_{K63} the targeted chain-linkage type constitutes 95.0%, 99.2%, and 99.5% of the total chain stock, respectively, while the directly translated di-Ub_{Linear} material exhibits 100% linear linkages. Similar evaluations of the tetramer stocks reveal linkage heterogeneities that are comparable to those of the shorter dimer species (Figure 3C); however, this constitutes the upper range of purity observed for K11-linked tetramer. Specifically, we have noted a slight propensity for the formation of mixed K11-K63 tetramers during tetra-Ub_{K11} preparation, as evidenced by occasional band doublets on SDS-PAGE gels of these stocks. Mass spectrometry data indicate that while the slower migrating band is uniformly K11-linked, the faster migrating band has substantial contaminating K63 linkages (Figure S3). No significant difference in K11-linkage fidelity is observed for full-length Ube2S enzyme versus the Ube2SΔC truncation mutant.

In sum, the ubiquitin-AQUA results verify the fidelity of the selected E2 enzymes used in the chain synthesis reactions, with less than a 0.8% error in linkage specificity for each addition of Ub protomer to K48 and K63 polymers, and affirm the high quality of the prepared chain stocks. While K11-linked chains show less linkage uniformity than the other chain types, the purity of these stocks has been of sufficiently high quality to enable structure determination and biophysical studies (Matsumoto et al., 2010). Recent work detailing the molecular mechanism of the Ube2S enzyme reveals that the substrate-assisted catalysis of K11-linked chain formation is mediated by weak electrostatic interactions (Wickliffe et al., 2011), suggesting that pH may play a role in specificity. We tested the effect of pH on linkage specificity and found that Ube2SΔC formed fewer K63-linked chains at higher pH (Figures S3E and S3F). Finally, due to the general reproducibility of the ubiquitin-AQUA results over multiple chain syntheses, we do not routinely perform this analysis on our prepared chain stocks unless we are attempting to incorporate previously untested forms of mutant or labeled Ub into the polyubiquitin chains.

Custom Synthesis and Application of Fluorescently Labeled Ubiquitin Chains

Given the utility of the purchased, fluorescein-labeled Ub in the E1 and E2 activity assays used to validate our enzyme stocks, we developed a methodology for synthesizing a variety of

fluorescently labeled Ub reagents. In our protocol, we first express and purify a mutant Ub construct, in which the first methionine residue is changed to a cysteine (Ub-M1C), as described above for wild-type Ub. Ub-M1C is then reacted with a maleimide-conjugated dye (e.g., fluorescein or Alexa-660) overnight, the reaction quenched with reducing agent and the protein separated from free dye by size exclusion chromatography. As wild-type Ub contains no native cysteines, Ub-M1C is selectively labeled at the introduced, N-terminal cysteine residue. Purified, labeled Ub-M1C is flash frozen and stored at −80°C.

Inclusion of fluorescein-labeled (Ub-Fl) or Alexa-660-labeled (Ub-Alexa) protein in Ub stocks (approximately 1% labeled Ub) used for in vitro polyubiquitin reactions does not appear to affect the synthesis of K11-, K48-, or K63-linked polyubiqutin chains as compared with reactions using only unlabeled Ub (Figure 4A). Moreover, ubiquitin-AQUA analysis of fluorescently labeled chains reveals no significant alteration in linkage specificity as compared with unlabeled polymers (Figure 4B). Although additional dye types may not be as well tolerated as the fluorescein or Alexa moeities, potential labeling schemes using this methodology are theoretically limited only by the availability of free maleimide-dye reagents.

One key benefit of fluorescently labeled polyubiquitin chains is the enhancement in signal detection as compared with unlabeled reagents. The increased sensitivity provided by these fluorescent chains enables significant reagent savings over the material required for traditional Coomassie staining of SDS-PAGE samples (Figure 4C). While western blot analysis still remains a more sensitive technique, the use of fluorescently labeled Ub enables faster detection and fewer assay components than immunoblotting protocols.

To further demonstrate the utility of these fluorescent ubiquitin chain reagents, we prepared K48- and K63-linked tetramers labeled with fluorescein and Alexa-660, respectively. Equimolar mixtures of the tetra-Ub-Fl_{K48} and tetra-Ub-$Alexa_{K63}$ chains were treated with the deubiquitinating enzyme, TRABID, and the resulting samples evaluated by fluorescent SDS-PAGE (Figure 4D). As previously reported, TRABID preferentially cleaved the K63-linked chains (Alexa-660 fluorescence) over the K48-linked chains (fluorescein fluorescence) (Komander et al., 2009). Because each ubiquitin chain type is tagged with a different fluorophore, one can test for linkage specificity within a single reaction by monitoring the two distinct fluorescent signals. Furthermore, these fluorescent reagents allow for more complex and/or abundant reaction components that could complicate analysis when a universal protein detection method like Coomassie staining is used. Again, while the dye conjugates may not be universally tolerated in all reactions of biological interest, this methodology could prove useful for interrogating a wide range of ubiquitin interactions. The possibility of applying these reagents to other fluorescence-based applications such as FP and FRET further broadens their potential utility.

DISCUSSION

The biological significance of ubiquitin modification of proteins has made the ability to generate and characterize specific interactions with defined-linkage ubiquitin chains an important area of study. The gold standard approach in this field for generating dimer, trimer or tetramer ubiquitin has been the methodology developed by Pickart and co-workers involving capping ubiquitin building blocks through point mutations/insertions to force select chain connectivity (Pickart and Raasi, 2005). This approach has been used to study the structure and dynamics of both isolated polyubiquitin chains (dimers or trimers of K48- and K63-linked ubiquitin) as well as chains in complex with Ub-binding proteins of interest (Blankenship et al., 2009; Newton et al., 2008; Reyes-Turcu et al., 2008; Sato et al., 2008, 2009; Varadan et al., 2004, 2005; Zhang et al., 2009). While this method offers precise control that allows for the differential labeling of specific ubiquitin protomers within a longer chain, which is advantageous for NMR approaches, it is time consuming, requires expression of a variety of ubiquitin mutants, and generally provides low yields. In addition, the capping process results in chains that differ at various positions from wild-type polyubiquitin. These differences may have unintended consequences if they impact interactions of interest. Moreover, this method is limited to chain types for which E2 or E2/E3 combinations are available, which to date only includes K11-, K48-, and K63-linkages.

To address this limitation, J.W. Chin and colleagues (Virdee et al., 2010) developed a novel approach to generate linkage-specific ubiquitin dimers that chemically couples engineered Ub constructs and is thus entirely independent of E1 and E2 enzyme activities. In this method, a thioester version of the "donor" Ub is expressed as a Ub-intein fusion, which is then modified using conventional amine protection chemistry such that its lysines are incapable of accepting isopeptide bonds. The "acceptor" Ub protein is generated with a lysine-Boc at a specific lysine position using unnatural amino acids and amber codons and the remaining lysine residues are amine protected in the same manner as the donor Ub molecule. These two chemically modified Ub proteins are then linked by isopeptide coupling to generate the dimer of interest, with a single round of global amine deprotection resulting in the wild-type dimer. This approach has been successfully used to generate K29 and K6 ubiquitin dimers; however, the complexity of the approach and the relatively low yield of the accepting Ub with the lysine-BOC may limit its utility for making longer chains. Additionally, the chemical conditions of the final deprotection step require unfolding of the ubiquitin chain product—a process that may be compromised by the refolding inefficiencies of longer polyubiquitin chains and/or site-directed mutations that affect the ability of ubiquitin to refold properly.

Other recent chemical syntheses of ubiquitin dimers avoid the denaturation process by utilizing thiol-lysines and native chemical ligation methods to build Ub proteins with alternative lysine protection groups that enable milder deprotection schemes; however, these complex, multistep reactions limit overall product yield (El Oualid et al., 2010; Yang et al., 2010). Fewer steps are required for another recent, nondenaturing, chemical synthesis approach in which a "click reaction" is utilized to link an acceptor Ub with an alkyne-functionalized lysine to a donor Ub containing an azide-functionalized, C-terminal methionine substitution (Eger et al., 2010). As with the intein approach, this methodology requires a significant investment in tRNAs and non-natural amino acids that complicate the synthesis of these ubiquitin polymers and can limit final product yields. Moreover, while initial tests demonstrate that the resulting triazole-linked ubiquitin dimers

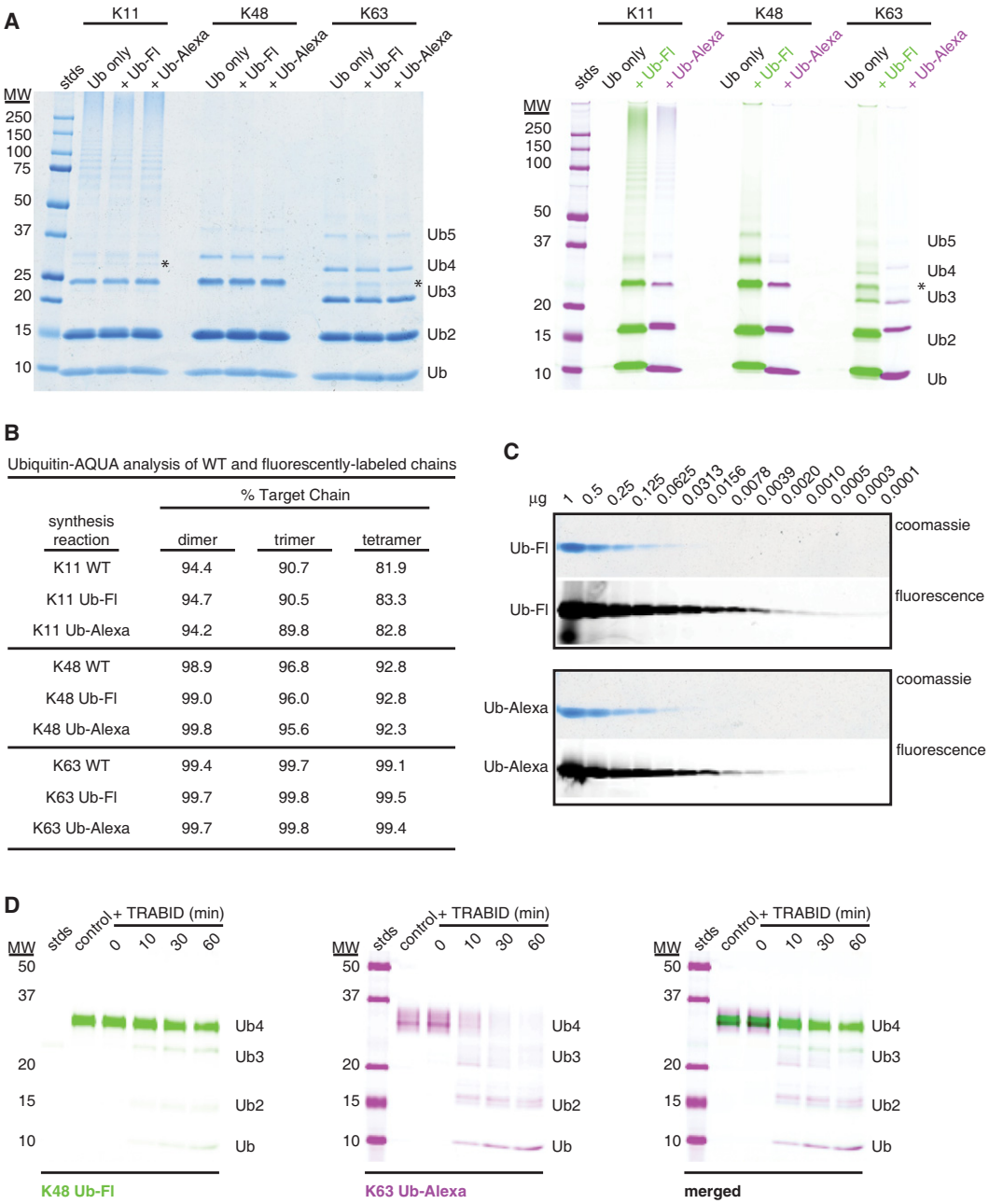

Figure 4. Application of Custom-Labeled, Fluorescent Polyubiquitin Chain Stocks

(A) Chain synthesis reactions are not visibly effected by inclusion of 1% Ub-Fl or Ub-Alexa in the Ub pool, with SDS-PAGE analysis of these reactions revealing no apparent differences with unlabeled Ub reactions by Coomassie staining. Fluorescent scans of the gel prior to staining reveal the presence of polyubiquitin chains labeled with fluorescein (green) or Alexa-660 (magenta) dye. MW markers prestained with blue dye are also visible in the Alexa channel. Asterisks denote small amounts of E2-Ub conjugates visible in the reaction.

(B) Ubiquitin-AQUA analysis of discrete bands from the synthesis reactions shown in (A) reveal no differences in linkage fidelity for labeled and unlabeled reactions (however, the % target chain values determined for bands from unprocessed synthesis reactions are routinely lower than those of purified stocks as shown in Figures 3B and 3C, with tetra-UbK_{11} showing the lower range of purity observed for this stock; see also Figure S1).

(C) Serial dilutions of Ub-Fl and Ub-Alexa stocks run on SDS-PAGE illustrate the increased sensitivity of the fluorescent labels over that of Coomassie staining.

(D) A time course of TRABID activity monitored by fluorescent imaging of a SDS-PAGE gel demonstrates the DUB's specificity for K63-linked chains (Alexa-660 labeled, magenta) over K48-linked chains (fluorescein labeled, green).

are competent substrates for E6-AP auto-ubiquitination assays, the lack of an isopeptide bond restricts the utility of these reagents (e.g., they may not be suitable for DUB recognition and cleavage reactions). Similar functional limitations exist for nonhydrolyzable ubiquitin-isopeptide isosteres chemically synthesized through oxime linkages (Shanmugham et al., 2010).

In contrast to the assortment of approaches discussed above, the method we describe here generates multimilligram quantities of highly pure, wild-type polyubiquitin chains using simple protein purification protocols and enzyme-driven syntheses that can be readily implemented in most protein structure/biophysics-based laboratories. Although this methodology is currently limited to select chain types (K11-, K48-, K63-linked and linear chains), it will likely be readily translatable to the production of K6-, K27-, K29- and K33-linked chains, as selective E2/E3 enzymes are identified and characterized for these other linkage types. To date, this approach has been used to produce defined pools of ubiquitin dimers, trimer, tetramers, and hexamers for biophysical and crystallographic studies (Bosanac et al., 2010; Dynek et al., 2010; Matsumoto et al., 2010) and K.C.D. and S.G.H. (unpublished data) and has proved to be robust to mutations that allow for fluorescent labeling of ubiquitin (e.g., Ub-M1C) or that affect ubiquitin function (e.g., I44A). Indeed, it is the ability to make specifically mutated chains that will enable detailed analyses of both intrachain and intermolecular interactions, which are critical to our understanding of how distinct polyubiquitin chain signals are interpreted by the cell. Custom synthesis of fluorescently labeled chains should also provide powerful tools to investigate questions of ubiquitin biology, enabling biochemical and biophysical studies that are intractable with currently available reagents.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification of Ubiquitin, Linear Ubiquitin Chains, and Ubiquitination Enzymes

We employed Genescript to synthesize genes encoding di-Ub_{Linear} (M1-G152) and tetra-Ub_{Linear} (M1-G304). Because of highly repetitive elements, the sequences were first codon optimized for efficient cloning and expression, then synthesized with NcoI and BamHI restriction sites to enable cloning into a pUC57 vector. We subsequently subcloned the genes into the same pET-15b bacterial expression plasmid (Invitrogen) as our monomeric Ub construct (M1-G76). A linear trimeric ubiquitin gene (M1-G228) was amplified from the tetrameric expression construct and subcloned into the pET-15b vector, as well. All constructs (Ub, di-Ub_{Linear}, tri-Ub_{Linear} and tetra-Ub_{Linear}) contain a thrombin-cleavable, N-terminal His_6-tag (MGSSHHHHHHSSGLVPR/GSH) and were expressed and purified as previously described for monomeric Ub (Bosanac et al., 2010). Briefly, constructs were transformed into BL21 pLysS cells, which were grown at 37°C in TB media buffered with 100 mM MOPS (pH 7.3) to an OD600 of 1.5–2.0 and the cultures cooled to room temperature prior to induction with 0.5 mM IPTG. Cells were harvested and frozen after overnight growth at 16°C. Cell paste was resuspended and homogenized in Ub lysis buffer (40 mM Tris [pH 8.0], 0.3 M NaCl) supplemented with EDTA-free Complete protease inhibitor tablets (Roche), the cells lysed by three successive passages through a M-110Y microfluidizer (Microfluidics, USA) and centrifuged to pellet cell debris. The resulting supernatant was passed over Ni-NTA resin (QIAGEN) and washed with Ub wash buffer (20 mM Tris [pH 7.2–7.5], 0.3M NaCl, 0.2 mM TCEP, 5 mM βME, 20 mM imidazole). Target protein was eluted with Ub wash buffer increased to 250 mM imidazole and dialyzed into Ub dialysis buffer (20 mM Tris [pH 8.0], 150 mM NaCl) in the presence of 1 unit of thrombin (CalBiochem) per 10 mg protein at 4°C overnight in order to remove the His_6-tag. Uncleaved protein was captured on Ni-NTA (QIAGEN) and the flow through was concentrated for size exclusion chromatography. In batches of about ~100 mg, ubiquitin monomer or each of the linear chains was injected on a HiLoad 26/60 Superdex 75 (GE Lifescience) in Ub SEC buffer (20 mM Tris [pH 7.5], 150 mM NaCl). Purity was monitored using SDS-PAGE and Coomassie staining. Here, it is important to note that the Tris-Glycine SDS gel loading buffer used did not contain reducing agent, the samples were not heated prior to gel loading, and the samples were mixed with loading buffer just prior to running the gel. Preparation of gel samples in this way limits the high molecular weight laddering artifacts sometimes observed in SDS-PAGE analyses of concentrated Ub stocks. Final recombinant ubiquitin pools were then concentrated and stored at 4°C (Ub) or −80°C (linear Ub chains).

UBE1 (M1-R1058) and Cdc34 (M1-S236) are pGEX6P-1 GST-tagged constructs with PreScission protease sites (Amersham). GST-UBE1 and GST-Cdc34 expressing bacteria were harvested by centrifugation and cell pellets resuspended in E1/Cdc34 lysis buffer (50 mM Tris-HCl [pH 7.5], 0.45M NaCl, 0.5% Triton X-100, 5 mM EDTA, 1 mM DTT) supplemented with protease inhibitor cocktail tablets (Roche). Samples were lysed as described above and the supernatant fraction was batch loaded onto glutathione Sepharose 4B resin (Amersham Biosciences) for 1 hr at 4°C. The loaded resin was washed with E1/Cdc34 lysis buffer followed by GST cleavage buffer (50 mM Tris-HCl [pH 7.5], 0.45 M NaCl, 5 mM EDTA, 1 mM DTT), then decanted into a conical tube and incubated with 40 μl PreScission Protease (Amersham) per milliliter of resin. The cleavage reaction was rotated overnight at 4°C, and then the resin was loaded onto a BioRad EconoPac column and the flow through was collected. The resin was washed with additional GST cleavage buffer and the pooled flow through and wash fractions were dialyzed overnight against either E1 dialysis buffer (50 mM HEPES [pH 8.0]) or Cdc34 dialysis buffer (50 mM HEPES [pH 8.0], 50 mM NaCl, 10% glycerol, 1 mM DTT). Samples were flash frozen in liquid nitrogen and stored at –80°C.

Uev1a (M1-N170) and UbcH13 (M1-I152) are His-tagged constructs with TEV protease sites. These proteins were expressed in BL21(DE3) cells and lysed in K63-E2 lysis buffer (20 mM Tris 80, 10% glycerol, 300 mM NaCl, 2 mM β-mercaptoethanol, 0.2 mM TCEP, 0.1% Triton X-100) and protease inhibitors according to procedures described above. Both constructs were purified by passage over a Ni-NTA (QIAGEN) affinity column using K63-E2 buffer A (20 mM Tris 8.0, 150 mM NaCl, 0.2 mM TCEP, 2 mM BME, 10 mM imidazole) and eluted using K63-E2 buffer A plus 250 mM imidazole. Following overnight incubation with TEV protease to remove the His tag, the proteins were passaged over a second Ni-NTA (QIAGEN) affinity column using the same buffers as above to remove any uncleaved material. Final purification was achieved by size exclusion chromatography using a Superdex 75 column (GE Lifescience) in 20 mM HEPES 7.5, 150 mM NaCl, and 0.5 mM TCEP. Proteins were quantified by A_{280}, mixed at a 1:1 molar ratio and flash frozen in liquid nitrogen for −80°C storage.

Ube2S (M1-L225) and Ube2SΔC (M1-G156) were cloned into the in-house pST239 vector with an N-terminal unizyme tag under the control of an alkaline phosphotase promoter and expressed in 58F3 cells [derived from W3110 with genotype Δ*fhuA*(Δ*tonA*) Δ*lon galE rpoHts*(*htpRts*) Δ*clpP lacIq* Δ*ompT*Δ (*nmpc-fepE*) Δ*slyD*] (Szeto et al., 2001). Small-volume cultures were grown overnight at 30°C and then diluted 100-fold into a phosphate-limiting medium to induce the alkaline phosphatase promoter. After 24 hr at 30°C with shaking, the cultures were centrifuged, and cell pellets harvested. Cells were resuspended in Ube2S lysis buffer 2 (20 mM phosphate [pH 8.0], 150 mM NaCl, 0.5 mM TCEP) supplemented with protease inhibitors. Following homogenation and lysis protocols detailed above, the samples were centrifuged and the soluble fraction was loaded onto a Ni-NTA (QIAGEN) column equilibrated in Ube2S buffer A (25 mM Tris-HCl [pH 80], 1 M NaCl, 20 mM imidazole). After washes with additional Ube2S buffer A, the Ube2S constructs were eluted with Ube2S elution buffer (25 mM Tris-HCl [pH 8.0], 0.5M NaCl, 250 mM imidazole). These fractions were pooled, concentrated, and further purified using size exclusion chromatography with a Superdex 75 column equilibrated (GE Lifescience) in Ube2S SEC buffer (20 mM HEPES [pH 7.5], 0.15 M NaCl, 0.5 mM TCEP). Fractions corresponding to monomeric Ube2S or Ube2SΔC were pooled and stored at –80°C.

Full-length TRABID (E3-E708) was expressed with an N-terminal His_6-tag in *T.ni* insect cells. Cell paste was resuspended in TRABID buffer A (50 mM Tris 8.0, 300 mM NaCl, 10 mM β-mercaptoethanol, 10 mM imidazole) supplemented with protease inhibitors and lysed as described above. Clarified lysate was affinity purified using Nickel Sepharose Fast Flow resin (QIAGEN) and eluted using TRABID buffer A with 250 mM imidazole. Eluted sample was cleaved with TEV protease overnight at 4°C, passed over a second nickel column and further purified over a Superdex 200 column (GE Amersham, USA) equilibrated in 20 mM Tris-HCl (pH 8.0), 0.5 M NaCl, and 2 mM β-mercaptoethanol. Fractions containing TRABID were pooled, concentrated, and stored at 4°C.

E1 Activation Assays and E2 Titrations

UBE1 activation was measured using a modified method derived from Pickart et al. and Haas et al., in which we replaced radioactive ^{125}I-G76A Ub with

fluorescently labeled Ub (Fl-ubiquitin) purchased from Invitrogen (Haas et al., 1982; Pickart et al., 1994). UBE1 (9–500 nM) was reacted with 150 nM Fl-ubiquitin, 2 mM ATP, 4 mM $MgCl_2$, and 50 mM Tris-HCl (pH 8.0) for 30 min at room temperature. Aliquots were transferred to a 386-well plate and read on a Victor 3 (Perkin Elmer) equipped with 485 ± 30 nm excitation and 535 ± 40 nm emission filters to measure fluorescence polarization of the samples (Figure 1C). To assess the reactions via gel-shift (Figure 1B), an equal volume of 50% glycerol was added to the remaining samples, which were then separated by SDS-PAGE and fluorescence signal detected with a blue (488 nm) laser and a 526 ± 20 nm emission filter using a Typhoon imager (GE Amersham). Band intensities were quantified using ImageQuant (GE Amersham). To evaluate E2 activity, we monitored the production of fluorescein-labeled polyubiqitin chains for a range of E2 concentrations. Small-scale test reactions (15 μl) were set up using similar conditions as described for the preparative synthesis reactions in the proceeding section, except that ~50 nM Fl-Ub (Invitrogen) was preincubated with the E1 enzyme for 15 min at room temperature prior to the addition of unlabeled Ub and E2 enzyme. Reactions were incubated overnight at 37°C and the resultant fluorescent species assessed by SDS-PAGE and FP measurements as described above. Data were plotted to assess optimal E2 concentrations for preparative synthesis reactions. These and all other plots presented here were generated using Kaleidagraph version 3.6 (Synergy Software).

Polyubiquitin Chain Synthesis and Purification

To generate preparative quantities of polyubiquitin chains, a reaction mixture containing ~50 mg of Ub at a final concentration of 1–1.5 mM, with 0.5 μM UBE and E1 activation buffer (50 mM Tris [pH 8.0], 10 mM ATP, 4–10 mM $MgCl_2$) and 0.6 mM DTT was incubated with either 30–50 μM Ube2SΔC, 5–10 μM Cdc34 or 2.5–5 μM Uev1a/UbcH13 for K11, K48, or K63 linkages, respectively (Komander et al., 2008; Pickart and Raasi, 2005; Wu et al., 2010). Exact E2 enzyme concentrations used depended upon the specific activity measured for particular E2 preparations (see Figure 1D). One should note that ATP regenerating systems (e.g., 20 mM creatine phosphate, 1.2 U/ml inorganic pyrophosphate, 1.2 U/ml creatine phosphokinase) are also compatible with these general synthesis conditions and may be used to drive chain production. Reactions were incubated overnight at 37°C, assessed by SDS-PAGE and quenched by 20-fold dilution into buffer A1 (50 mM ammonium acetate, 100 mM NaCl [pH 4.5]). Polyubiquitin chains of defined lengths were separated on a MonoS 10/100 GL column (GE LifeScience) with a 45 column volume gradient of buffers A1 and B (50 mM ammonium acetate [pH 4.5], 0.6 M NaCl). A portion of the dimeric ubiquitin was pooled from the MonoS column elution, concentrated, and exchanged into Ub SEC buffer, through dilution and reconcentration, to lower the salt concentration to 150 mM and increase the pH to 7.5. This dimer pool was then used to create sufficient tetramer chains utilizing the reaction conditions stated above, where dimer replaces monomeric ubiquitin as the starting material. The separation of these higher order chains was then accomplished using a 45 column volume gradient of buffers A2 (50 mM ammonium acetate [pH 4.5], 0.3M NaCl) and B. Each chain length was pooled separately and concentrated to less than 1 ml for injection onto a HiLoad 16/60 Superdex 75 (GE LifeScience) for final purification to homogeneity. The resulting chain stocks were concentrated, quantified, and stored at −80°C.

Preparing Fluorescently Tagged Ubiquitin and Polyubiquitin Chains

To produce labeled ubiquitin stocks, 2 mg of Ub-M1C was first reduced with ten molar excess of Tris(2-carboxyethyl)phosphine (TCEP) and then reacted with a 10-fold molar excess of flourescein-maleimide (Invitrogen) or Alexa-660-maleimide (Invitrogen) overnight at 4°C. The reactions were quenched with 2.9 mM β-mercaptoethanol, dialyzed into Ub SEC buffer and loaded onto a Sephacryl 100 column (GE Amersham) to remove unbound dye. Chain synthesis reactions containing 1% labeled Ub were carried out as described above with the following exceptions: 3 μM UBE1 was preincubated with either 15 μM Ub-Fl or Ub-Alexa in E1 activation buffer for 15 min at 37°C prior to the addition of unlabeled Ub and E2 enzyme(s) for overnight incubation at the same temperature. Reaction products were separated by SDS-PAGE and scanned using a Typhoon imager prior to Coomassie staining. Chains containing Ub-Fl were detected using the settings detailed above whereas chains labeled with Ub-Alexa were imaged using a red (630 nm) laser and a 670 ± 30 nm emission filter. Purified tetra-Ub-Fl_{K48} and tetra-Ub-$Alexa_{K63}$ stocks were prepared in a similar fashion; however, the order of the chain synthesis and labeling steps was reversed. Specifically, we first synthesized and purified tetrameric chains as described above, using a monomeric Ub starting pool that contained 10% Ub-M1C. This Ub pool was preincubated in chain synthesis reaction buffer for 5 min prior to addition of the remaining reaction components to reduce potential disulfide bonds formed between Ub-M1C molecules in the concentrated stock. Purified tetramers were then reacted with dye (tetra-UB_{K48} with fluorescein-maleimide and tetra-UbK_{63} with Alexa-660-maleimide) and purified as described above for monomeric Ub-M1C protein.

Quantitative Mass Spectrometry

The composition of Ub chains was determined by either multiple-reaction monitoring on a QTrap4000 or from high-resolution full MS scans on an LTQ-Orbitrap XL mass spectrometer, as recently described (Kirkpatrick et al., 2006; Phu et al., 2010). Briefly, purified Ub chain stocks or chain synthesis reactions were separated by SDS-PAGE and polyubiquitin bands subjected to in-gel trypsin digestion with 20 ng/μl trypsin solution (50 mM ammonium bicarbonate/5% acetonitrile). Isotope-labeled synthetic peptides of Ub and the branched Ub signature peptides (ggSP) were added to gel pieces prior to extracting the digested peptides. Extracted peptides were dried completely, resuspended and separated by reverse phase HPLC using standard water/acetonitrile/formic acid based gradients. Since AQUA peptide stock concentrations can vary systematically from their measured concentrations following preparation of diluted mixtures, separate di-Ub bands of multiple linkages were analyzed in parallel with each experiment as controls. The total amount of Ub in a control di-Ub band was initially determined as the average of independent measurements made for unbranched peptides and ggSP from the K11, K33, K48, and K63 loci. Correction factors were determined for ggSPs based on the defined 1:2 stoichiometry of linkage to total Ub for a given dimer, and corrected ggSP values reintroduced to generate revised total Ub amounts. The process was repeated through two iterations. The abundance of a given linkage is reported as percent total Ub chains, representing the calculated abundance of a given ggSP relative to the sum of ggSP from all chains.

DUB Assay

TRABID was diluted to 10 μM in 25 mM Tris (pH 7.5), 150 mM NaCl, and 10 mM DTT and preincubated for 20 min at room temperature. A final concentration of 1 μM TRABID was incubated with a mixture of 500 nM tetra-Ub_{K48} and 500 nM tetra-Ub_{K63} labeled with approximately 10% fluorescein, and 10% Alexa-660, respectively, in assay buffer (50 mM Tris [pH 7.5], 50 mM NaCl, 2 mM DTT) at room temperature. Aliquots were removed at specific time points to an equivalent volume of 2 × SDS sample buffer and heated at 94°C for 5 min in order to stop the reaction. Samples (4 μl) were separated by SDS-PAGE and imaged by Typhoon scanner prior to Coomassie staining as described above.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this article online at doi:10.1016/j.str.2011.06.010.

ACKNOWLEDGMENTS

We thank Adam Eldridge and Brigitte Maurer for their help generating GST-Cdc34 and His_6-tri-Ub_{Linear} expression constructs, respectively, the DNA Sequencing facility for verifying the integrity of all expression constructs, and Matthew Bentley, Vishva Dixit, Michael Rape, and Ron Hays for helpful discussions. The authors are employees of Genentech, Inc.

Received: April 22, 2011
Revised: June 24, 2011
Accepted: June 25, 2011
Published: August 9, 2011

REFERENCES

Baboshina, O.V., and Haas, A.L. (1996). Novel multiubiquitin chain linkages catalyzed by the conjugating enzymes E2EPF and RAD6 are recognized by 26 S proteasome subunit 5. J. Biol. Chem. *271*, 2823–2831.

Blankenship, J.W., Varfolomeev, E., Goncharov, T., Fedorova, A.V., Kirkpatrick, D.S., Izrael-Tomasevic, A., Phu, L., Arnott, D., Aghajan, M., Zobel, K., et al. (2009). Ubiquitin binding modulates IAP antagonist-stimulated proteasomal degradation of c-IAP1 and c-IAP2(1). Biochem. J. *417*, 149–160.

Bosanac, I., Wertz, I.E., Pan, B., Yu, C., Kusam, S., Lam, C., Phu, L., Phung, Q., Maurer, B., Arnott, D., et al. (2010). Ubiquitin binding to A20 ZnF4 is required for modulation of NF-kB signaling. Mol. Cell. *40*, 548–557.

Chau, V., Tobias, J.W., Bachmair, A., Marriott, D., Ecker, D.J., Gonda, D.K., and Varshavsky, A. (1989). A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. Science *243*, 1576–1583.

Chen, Z., and Pickart, C.M. (1990). A 25-kilodalton ubiquitin carrier protein (E2) catalyzes multi-ubiquitin chain synthesis via lysine 48 of ubiquitin. J. Biol. Chem. *265*, 21835–21842.

Chen, Z.J., and Sun, L.J. (2009). Nonproteolytic functions of ubiquitin in cell signaling. Mol. Cell *33*, 275–286.

Chen, Z.J., Niles, E.G., and Pickart, C.M. (1991). Isolation of a cDNA encoding a mammalian multiubiquitinating enzyme (E225K) and overexpression of the functional enzyme in Escherichia coli. J. Biol. Chem. *266*, 15698–15704.

Cook, W.J., Jeffrey, L.C., Carson, M., Chen, Z., and Pickart, C.M. (1992). Structure of a diubiquitin conjugate and a model for interaction with ubiquitin conjugating enzyme (E2). J. Biol. Chem. *267*, 16467–16471.

Cook, W.J., Jeffrey, L.C., Kasperek, E., and Pickart, C.M. (1994). Structure of tetraubiquitin shows how multiubiquitin chains can be formed. J. Mol. Biol. *236*, 601–609.

Dynek, J.N., Goncharov, T., Dueber, E.C., Fedorova, A.V., Izrael-Tomasevic, A., Phu, L., Helgason, E., Fairbrother, W.J., Deshayes, K., Kirkpatrick, D.S., and Vucic, D. (2010). c-IAP1 and UbcH5 promote K11-linked polyubiquitination of RIP1 in TNF signalling. EMBO J. *29*, 4198–4209.

Eddins, M.J., Varadan, R., Fushman, D., Pickart, C.M., and Wolberger, C. (2007). Crystal structure and solution NMR studies of Lys48-linked tetraubiquitin at neutral pH. J. Mol. Biol. *367*, 204–211.

Eger, S., Scheffner, M., Marx, A., and Rubini, M. (2010). Synthesis of defined ubiquitin dimers. J. Am. Chem. Soc. *132*, 16337–16339.

El Oualid, F., Merkx, R., Ekkebus, R., Hameed, D.S., Smit, J.J., de Jong, A., Hilkmann, H., Sixma, T.K., and Ovaa, H. (2010). Chemical synthesis of ubiquitin, ubiquitin-based probes, and diubiquitin. Angew. Chem. Int. Ed. Engl. *49*, 10149–10153.

Garnett, M.J., Mansfeld, J., Godwin, C., Matsusaka, T., Wu, J., Russell, P., Pines, J., and Venkitaraman, A.R. (2009). UBE2S elongates ubiquitin chains on APC/C substrates to promote mitotic exit. Nat. Cell Biol. *11*, 1363–1369.

Gerlach, B., Cordier, S.M., Schmukle, A.C., Emmerich, C.H., Rieser, E., Haas, T.L., Webb, A.I., Rickard, J.A., Anderton, H., Wong, W.W., et al. (2011). Linear ubiquitination prevents inflammation and regulates immune signalling. Nature *471*, 591–596.

Harper, J.W., and Schulman, B.A. (2006). Structural complexity in ubiquitin recognition. Cell *124*, 1133–1136.

Haas, A.L., Warms, J.V.B., Hershko, A., and Rose, I.A. (1982). Ubiquitin-activating enzyme. Mechanism and role in protein-ubiquitin conjugation. J. Biol. Chem. *257*, 2543–2548.

Ikeda, F., Deribe, Y.L., Skånland, S.S., Stieglitz, B., Grabbe, C., Franz-Wachtel, M., van Wijk, S.J., Goswami, P., Nagy, V., Terzic, J., et al. (2011). SHARPIN forms a linear ubiquitin ligase complex regulating NF-κB activity and apoptosis. Nature *471*, 637–641.

Ikeda, F., and Dikic, I. (2008). Atypical ubiquitin chains: new molecular signals. 'Protein Modifications: Beyond the Usual Suspects' review series. EMBO Rep. *9*, 536–542.

Iwai, K., and Tokunaga, F. (2009). Linear polyubiquitination: a new regulator of NF-kappaB activation. EMBO Rep. *10*, 706–713.

Jin, L., Williamson, A., Banerjee, S., Philipp, I., and Rape, M. (2008). Mechanism of ubiquitin-chain formation by the human anaphase-promoting complex. Cell *133*, 653–665.

Kirkpatrick, D.S., Hathaway, N.A., Hanna, J., Elsasser, S., Rush, J., Finley, D., King, R.W., and Gygi, S.P. (2006). Quantitative analysis of in vitro ubiquitinated cyclin B1 reveals complex chain topology. Nat. Cell Biol. *8*, 700–710.

Komander, D., Lord, C.J., Scheel, H., Swift, S., Hofmann, K., Ashworth, A., and Barford, D. (2008). The structure of the CYLD USP domain explains its specificity for Lys63-linked polyubiquitin and reveals a B box module. Mol. Cell *29*, 451–464.

Komander, D., Reyes-Turcu, F., Licchesi, J.D.F., Odenwaelder, P., Wilkinson, K.D., and Barford, D. (2009). Molecular discrimination of structurally equivalent Lys 63-linked and linear polyubiquitin chains. EMBO Rep. *10*, 466–473.

Lo, Y.C., Lin, S.C., Rospigliosi, C.C., Conze, D.B., Wu, C.J., Ashwell, J.D., Eliezer, D., and Wu, H. (2009). Structural basis for recognition of diubiquitins by NEMO. Mol. Cell *33*, 602–615.

Matsumoto, M.L., Wickliffe, K.E., Dong, K.C., Yu, C., Bosanac, I., Bustos, D., Phu, L., Kirkpatrick, D.S., Hymowitz, S.G., Rape, M., et al. (2010). K11-linked polyubiquitination in cell cycle control revealed by a K11 linkage-specific antibody. Mol. Cell *39*, 477–484.

Newton, K., Matsumoto, M.L., Wertz, I.E., Kirkpatrick, D.S., Lill, J.R., Tan, J., Dugger, D., Gordon, N., Sidhu, S.S., Fellouse, F.A., et al. (2008). Ubiquitin chain editing revealed by polyubiquitin linkage-specific antibodies. Cell *134*, 668–678.

Phu, L., Izrael-Tomasevic, A., Matsumoto, M.L., Bustos, D., Dynek, J.N., Fedorova, A.V., Bakalarski, C.E., Arnott, D., Deshayes, K., Dixit, V.M., et al. (2010). Improved quantitative mass spectrometry methods for characterizing complex ubiquitin signals. Mol. Cell Proteomics *10*, M110.003756.

Pickart, C.M., and Fushman, D. (2004). Polyubiquitin chains: polymeric protein signals. Curr. Opin. Chem. Biol. *8*, 610–616.

Pickart, C.M., and Raasi, S. (2005). Controlled synthesis of polyubiquitin chains. Methods Enzymol. *399*, 21–36.

Pickart, C.M., Kasperek, E.M., Beal, R., and Kim, A. (1994). Substrate properties of site-specific mutant ubiquitin protein (G76A) reveal unexpected mechanistic features of ubiquitin-activating enzyme (E1). J. Biol. Chem. *269*, 7115–7123.

Piotrowski, J., Beal, R., Hoffman, L., Wilkinson, K.D., Cohen, R.E., and Pickart, C.M. (1997). Inhibition of the 26 S proteasome by polyubiquitin chains synthesized to have defined lengths. J. Biol. Chem. *272*, 23712–23721.

Rahighi, S., Ikeda, F., Kawasaki, M., Akutsu, M., Suzuki, N., Kato, R., Kenshe, T., Uejima, T., Bloor, S., Komander, D., et al. (2009). Specific recognition of linear ubiquitin chains by NEMO is important for NF-kB activation. Cell *136*, 1098–1109.

Reyes-Turcu, F.E., Shanks, J.R., Komander, D., and Wilkinson, K.D. (2008). Recognition of polyubiquitin isoforms by the multiple ubiquitin binding modules of isopeptidase T. J. Biol. Chem. *283*, 19581–19592.

Sato, Y., Yoshikawa, A., Yamagata, A., Mimura, H., Yamashita, M., Ookata, K., Nureki, O., Iwai, K., Komada, M., and Fukai, S. (2008). Structural basis for specific cleavage of Lys 63-linked polyubiquitin chains. Nature *455*, 358–362.

Sato, Y., Yoshikawa, A., Yamashita, M., Yamagata, A., and Fukai, S. (2009). Structural basis for specific recognition of Lys 63-linked polyubiquitin chains by NZF domains of TAB2 and TAB3. EMBO J. *28*, 3903–3909.

Shanmugham, A., Fish, A., Luna-Vargas, M.P., Faesen, A.C., El Oualid, F., Sixma, T.K., and Ovaa, H. (2010). Nonhydrolyzable ubiquitin-isopeptide isosteres as deubiquitinating enzyme probes. J. Am. Chem. Soc. *132*, 8834–8835.

Szeto, W., Jiang, W., Tice, D.A., Rubinfeld, B., Hollingshead, P.G., Fong, S.E., Dugger, D.L., Pham, T., Yansura, D.G., Wong, T.A., et al. (2001). Overexpression of the retinoic acid-responsive gene Stra6 in human cancers and its synergistic induction by Wnt-1 and retinoic acid. Cancer Res. *61*, 4197–4205.

Tokunaga, F., Nakagawa, T., Nakahara, M., Saeki, Y., Taniguchi, M., Sakata, S., Tanaka, K., Nakano, H., and Iwai, K. (2011). SHARPIN is a component of

the NF-κB-activating linear ubiquitin chain assembly complex. Nature *471*, 633–636.

Tseng, P., Matsuzawa, A., Zhang, W., Mino, T., Vignali, D., and Karin, M. (2010). Different modes of ubiquitination of the adaptor TRAF3 selectively activate the expression of type I interferons and proinflammatory cytokines. nature immunology *11*, 70–76.

Varadan, R., Assfalg, M., Haririnia, A., Raasi, S., Pickart, C., and Fushman, D. (2004). Solution conformation of Lys63-linked di-ubiquitin chain provides clues to functional diversity of polyubiquitin signaling. J. Biol. Chem. *279*, 7055–7063.

Varadan, R., Assfalg, M., Raasi, S., Pickart, C., and Fushman, D. (2005). Structural determinants for selective recognition of a Lys48-linked polyubiquitin chain by a UBA domain. Mol. Cell *18*, 687–698.

Varadan, R., Walker, O., Pickart, C., and Fushman, D. (2002a). Structural properties of polyubiquitin chains in solution. J. Mol. Biol. *324*, 637–647.

Varadan, R., Walker, O., Pickart, C.M., and Fushman, D. (2002b). Structural properties of polyubiquitin chains in solution. J. Mol. Biol. *324*, 637–647.

Virdee, S., Ye, Y., Nguyen, D.P., Komander, D., and Chin, J.W. (2010). Engineered diubiquitin synthesis reveals Lys29-isopeptide specificity of an OTU deubiquitinase. Nat. Chem. Biol. *6*, 750–757.

Wickliffe, K.E., Lorenz, S., Wemmer, D.E., Kuriyan, J., and Rape, M. (2011). The mechanism of linkage-specific ubiquitin chain elongation by a single-subunit E2. Cell *144*, 769–781.

Williamson, A., Wickliffe, K.E., Mellone, B.G., Song, L., Karpen, G.H., and Rape, M. (2009). Identification of a physiological E2 module for the human anaphase-promoting complex. Proc. Natl. Acad. Sci. USA *106*, 18213–18218.

Wu, T., Merbl, Y., Huo, Y., Gallop, J.L., Tzur, A., and Kirschner, M.W. (2010). UBE2S drives elongation of K11-linked ubiquitin chains by the anaphase-promoting complex. Proc. Natl. Acad. Sci. USA *107*, 1355–1360.

Xu, P., Duong, D.M., Seyfried, N.T., Cheng, D., Xie, Y., Robert, J., Rush, J., Hochstrasser, M., Finley, D., and Peng, J. (2009). Quantitative proteomics reveals the function of unconventional ubiquitin chains in proteasomal degradation. Cell *137*, 133–145.

Yang, R., Pasunooti, K.K., Li, F., Liu, X.W., and Liu, C.F. (2010). Synthesis of K48-linked diubiquitin using dual native chemical ligation at lysine. Chem. Commun. (Camb.) *46*, 7199–7201.

Yao, T., and Cohen, R.E. (2000). Cyclization of polyubiquitin by the E2-25K ubiquitin conjugating enzyme. J. Biol. Chem. *275*, 36862–36868.

Zhang, N., Wang, Q., Ehlinger, A., Randles, L., Lary, J.W., Kang, Y., Haririnia, A., Storaska, A.J., Cole, J.L., Fushman, D., and Walters, K.J. (2009). Structure of the s5a:k48-linked diubiquitin complex and its interactions with rpn13. Mol. Cell *35*, 280–290.